SHORT COMMUNICATION

Detection of *Coxiella burnetii* by Nested PCR in Bulk Milk Samples from Dairy Bovine, Ovine, and Caprine Herds in Iran

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Impact

- Q-fever is a zoonosis caused by an intracellular rickettsial bacterium *Coxiella burnetii* with worldwide distribution.
- Among livestock, dairy cattle, sheep, and goats are the major reservoirs of *C. burnetii*; often the infected animals are asymptomatic.
- Raw milk or dairy products that are produced by unpasteurized milk (cheese) may contain virulent *C. burnetii.*

Keywords:
Coxiella burnetii; PCR; milk; ruminants; Iran

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Summary

The epidemiology of Q-fever in Iran is essentially unknown. This study was conducted to determine the prevalence rate of *Coxiella burnetii* in bulk milk samples from dairy bovine, ovine, and caprine herds in Chaharmahal va Bakhtiari province, Iran. In this study, 376 bulk milk samples from 79 dairy bovine, ovine, and caprine herds were tested for *C. burnetii* using a nested PCR assay. The animals whose milk samples collected for this study were clinically healthy. In total, 13 of 210 (6.2%) bovine milk samples were positive; the positive samples originated from 5 of 28 (17.9%) commercial dairy herds. All 110 ovine bulk milk samples from 31 sheep breeding farms were negative and only 1 of 56 (1.8%) caprine bulk milk samples from 20 goat breeding farms was positive for *C. burnetii*. Although no extensive prevalence study was undertaken, the results of this study indicate that clinically healthy cattle are important sources of *C. burnetii* infection in Iran. To our knowledge, this study is the first report of direct identification of *C. burnetii* by PCR in bulk milk samples from dairy bovine and caprine herds in Iran. Further intensive prevalence studies on *Coxiella* infection among farmers, milk-processing workers, veterinarians, and slaughterhouse workers and on possible dangers of dairy products will be needed to elucidate the epidemiology of Q fever in Iran.

Introduction

Q-fever is a zoonosis caused by an intracellular rickettsial bacterium with worldwide distribution, *Coxiella burnetii* (Thomas et al., 1995). A wide variety of animals can be infected with *C. burnetii* including mammals such as ruminants, dogs, cats, non-human primates, wild rodents, small mammals, big game wildlife, and non-mammals such as reptiles, amphibians, birds, fish, and ticks (Parker et al., 2006). Among livestock, dairy cattle, sheep, and goats are the major reservoirs of *C. burnetii* (Kim et al., 2005) and are more frequently related to outbreaks of human Q fever than other animal species (Tissot Dupont et al., 1992; Berri et al., 2003). The uterus and mammary

glands are primary sites of infection in the chronic phase of *C. burnetii*. Shedding of *C. burnetii* into the environment occurs mainly during parturition by birth products, particularly the placenta but may also be shed via milk, feces, vaginal mucus, urine, and semen (Maurin and Raoult, 1999; Kim et al., 2005; Guatteo et al., 2006).

Q-fever is essentially an airborne disease. Infections occur after inhalation of aerosols generated from infected animal's placenta or body fluids (Arricau-Bouvery and Rodolakis, 2005). While infection from commercial milk is unlikely because of the pasteurization process, raw milk or dairy products that are produced by unpasteurized milk (cheese) may contain virulent *C. burnetii* (Berri et al., 2000). Drinking contaminated milk has induced sero-conversion in human volunteers without clinical signs (Arricau-Bouvery and Rodolakis, 2005).

The epidemiology of Q-fever in Iran is essentially unknown, and to the authors' knowledge, the prevalence rate of *C. burnetii* in ruminants in Iran has never been reported. The objective of this study was to determine the prevalence rate of *C. burnetii* in bulk milk samples from dairy bovine, caprine, and ovine herds in Chaharmahal va Bakhtiari province, Iran using a nested PCR assay. Although, governmental regulation of milk pasteurization and sanitation in dairy processing plants has been in existence in Iran for many years, direct sale of unpasteurized milk and dairy products from producers to the consumer is not uncommon in many regions including Chaharmahal va Bakhtiari province.

Materials and Methods

Collection of samples

Bovine, ovine, and caprine herds were randomly selected in Chaharmahal va Bakhtiari province, Iran. This province is located in the central and southern part of Iran with about 850 000 inhabitants. From February to October 2008 a total of 210 bovine bulk milk samples were collected from 28 commercial dairy herds (4–6 years old Holstein cows). From March to April 2008 a total of 110 ovine bulk milk samples were collected from 31 sheep breeding farms (2–10 years old Lori-Bakhtiari breed) and from September to October 2008 a total of 56 caprine bulk milk samples were collected from 20 goat breeding farms (2–10 years old Lori-Bakhtiari breed) (Table 1). Bovine, ovine, and caprine herds were strictly separated from each other and the animals were kept in free-stall housing. *Coxiella burnetii* infection status of the herds was unknown prior to this study and there was no established surveillance or management targeting *C. burnetii* control. The animals whose milk samples collected for this study were clinically healthy and the milk samples showed physical (color, pH, and density) consistency. The samples were immediately transported to the laboratory in a cooler with ice packs and were processed within an hour of collection.

PCR detection of *C. burnetii*

Coxiella burnetii was isolated from milk samples by centrifuging and removing cream and milk layers as described previously (Berri et al., 2003). Purification of DNA was achieved using a Genomic DNA purification kit (Fermentas, GmbH, Germany) according to the manufacturer's instructions and the total DNA was measured at 260 nm optical density according to the method described by Sambrook and Russell (2001).

All oligonucleotide primers were obtained from a commercial source (Cinna Gen, Iran). The nested PCR assay used to screen for *C. burnetii* was designed from the nucleotide sequence of the *com1* gene encoding a 27-kD outer membrane protein (OMP) as previously described (Zhang et al., 1998) and the amplification was carried according to the method described else where (Fretz et al., 2007). For the nested PCR assay with primers OMP1-OMP2 and OMP3-OMP4, the first amplification was performed in a total volume of 25 μl containing 5 μl of DNA sample, 0.5 mM MgCl2, 0.2 mM (each) dNTPs, 1 μM primer OMP1, 1 μM primer OMP2, and 0.5 U/reaction of Taq DNA polymerase (Roche Applied Science, Mannheim, Germany).

The PCR assay was performed at 94°C for 4 min and then for 30 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min in a DNA thermal cycler (Master Cycler Gradiant, Eppendrof, Germany). In the second amplification, the reaction was performed in a total volume of 25 μl containing 2 μl of DNA sample, 0.5 mM MgCl2, 0.2 mM (each) dNTPs, 0.8 μM primer OMP3, 0.8 μM

Table 1. Prevalence of *Coxiella burnetii* in bulk milk samples from dairy bovine, ovine, and caprine herds in Chaharmahal va Bakhtiari province, Iran

	No. herds in the study region	No. herds studied	No. samples per herd	No. milk samples	No. (%) *Coxiella burnetii* positive samples
Cow	95	28	6–9	210	13 (6.2)
Sheep	120	31	2–4	110	0 (0.0)
Goat	70	20	2–4	56	1 (1.8)

primer OMP4, and 0.5 U/reaction of Taq DNA polymerase. The PCR assay was performed at 95°C for 4 min and then for 30 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min. The PCR-amplified products (OMP1-OMP2: 501 bp; OMP3-OMP4: 438 bp) were examined by electrophoresis in a 1.5% agarose gel, stained with a 1% solution of ethidium bromide, and examined under UV illumination. In this study, *C. burnetii* DNA (Serial Number: 3154; Genekam Biotechnology AG, Duisburg, Germany) and DNase free water were used as the positive and negative controls, respectively.

Results and Discussion

In this study, a total of 376 bulk milk samples from 79 dairy bovine, ovine, and caprine herds in Chaharmahal va Bakhtiari province of Iran were tested for *C. burnetii* using a nested PCR assay (Fig. 1). In total, 13 of 210 (6.2%; 95% confidence interval, 0.0–0.1%) bovine milk samples were positive (Table 1). The positive samples were from 5 of 28 (17.9%; 95% confidence interval, 0.0–1.0%) commercial dairy herds. Eleven of 13 positive bulk milk samples were from three dairy herds (herd number 3, 12, and 28) with 3, 4, and 4 positive samples, respectively (Table 2). This result is similar to a recent report in Switzerland that showed a prevalence of *C. burnetii* of 4.7% (n = 359) in bovine bulk tank milk samples using a nested PCR assay (Fretz et al., 2007). Another study from the USA showed a very high prevalence of *C. burnetii* (>90%) in bulk tank milk samples from dairy herds over a 3-year period (Kim et al., 2005).

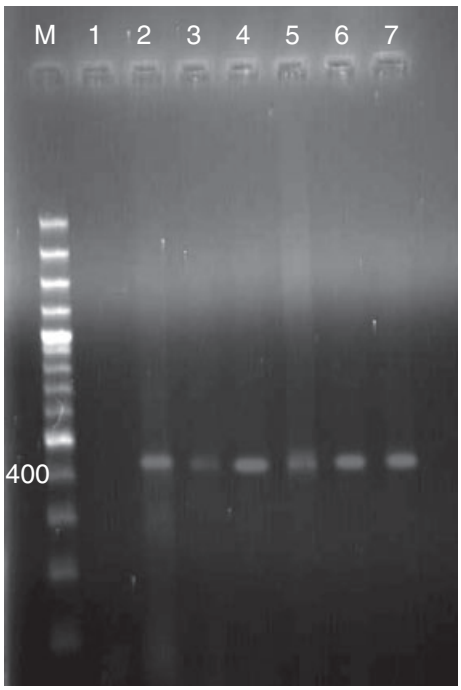

Fig. 1. Electropherogram of the amplification products of the nested polymerase chain reaction assay. M, 100 bp DNA ladder; lane1, negative control; lane 2, positive control; lanes 3–7, *Coxiella burnetii* positive milk samples from dairy bovine herds.

All 110 ovine bulk milk samples from 31 sheep breeding farms were negative and only 1 of 56 (1.8%; 95% confidence interval, 0.0–0.1%) caprine bulk milk samples from 20 goat breeding farms was positive for *C. burnetii*. In a recent study in Switzerland, all 81 ovine and 39 caprine bulk milk samples were negative for *C. burnetii* using a nested PCR assay (Fretz et al., 2007). In another study conducted in Turkey, 3.5% of single milk samples from 400 sheep of 22 flocks were positive for *C. burnetii* by a PCR assay (Öngör et al., 2004).

Because *C. burnetii* may be shed by other routes such as vaginal mucus, feces, urine, placenta, or birth fluids, testing animal based on only milk sample can be lead to misclassify the status of the animal (Guatteo et al., 2006). In fact, the differences between the prevalence of *C. burnetii* in bovine, ovine, and caprine milk samples found in this study may be because of the different routes of shedding *C. burnetii* present in these animals. Ovine shed *C. burnetii* mainly in feces and vaginal mucus, whereas bovine shed *C. burnetii* mainly in milk. Caprine excrete *C.* burnetii in their vaginal discharges, feces, and milk (Rodolakis et al., 2007). Furthermore, the infected animals may not persistently shed *C. burnetii*. Shedding of *C. burnetii* by infected animals occurs mainly during parturition and lactation. Therefore, detection of *C. burnetii* in bulk tank milk greatly depends on the sampling time. The use of repeated sampling can reduce the likelihood of falsely classifying a herd as *C. burnetii* negative (Guatteo et al., 2007). The findings of this study are limited to PCR-based detection of *C. burnetii* DNA in bulk milk samples, so we are unable to speculate on the viability of organisms in milk samples, or on the sensitivity and specificity of the nested PCR assay compared to other diagnostic methods.

Although no extensive prevalence study was undertaken, the results of this study indicate that clinically healthy cattle are important sources of *C. burnetii* infection in Iran. Therefore, to prevent the spread of infection in animal and human populations, control of bovine coxiellosis should be instituted. The present results also suggest that the bulk tank milk, which is easy and inexpensive to collect, could be used to assess, on a larger scale at a low cost, the efficiency of control schemes aimed at controlling and/or preventing *Coxiella* shedding in dairy herds.

To our knowledge, this study is the first report of direct identification of *C. burnetii* by PCR in bulk milk

Table 2. Distribution of *Coxiella burnetii* bulk milk-positive samples from 5 dairy bovine herds during February–October, 2008 in Chaharmahal va Bakhtiari province, Iran

Dairy herd no.	February–March	April–June	July–August	September–October	Total of positive samples
3	+	+	+	−	3
12	+	+, +	+	−	4
14	−	−	−	+	1
17	−	−	−	+	1
28	+, +	+	+	−	4

samples from dairy bovine and caprine herds in Iran. Further intensive prevalence studies on *Coxiella* infection among farmers, milk-processing workers, veterinarians, and slaughterhouse workers and on possible dangers of dairy products will be needed to elucidate the epidemiology of Q fever in Iran.

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